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Fusarium Tri8 Encodes a Trichothecene C-3 Esterase

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Mutant strains of *Fusarium graminearum* Z3639 produced by disruption of *Tri8* were altered in their ability to biosynthesize 15-acetyldeoxynivalenol and instead accumulated 3,15-diacetyldeoxynivalenol, 7,8-dihydroxycalonectrin, and calonectrin. *Fusarium sporotrichioides* NRRL3299 *Tri8* mutant strains accumulated 3-acetyl T-2 toxin, 3-acetyl neosolaniol, and 3,4,15-triacetoxyscirpenol rather than T-2 toxin, neosolaniol, and 4,15-diacetoxyscirpenol. The accumulation of these C-3-acetylated compounds suggests that *Tri8* encodes an esterase responsible for deacetylation at C-3. This gene function was confirmed by cell-free enzyme assays and feeding experiments with yeast expressing *Tri8*. Previous studies have shown that *Tri101* encodes a C-3 transacetylase that acts as a self-protection or resistance factor during biosynthesis and that the presence of a free C-3 hydroxyl group is a key component of *Fusarium* trichothecene phytotoxicity. Since *Tri8* encodes the esterase that removes the C-3 protecting group, it may be considered a toxicity factor.

Trichothecenes are sesquiterpene epoxides that are produced by several *Fusarium* species. *Fusarium* trichothecenes can be divided into two groups based on the substitution of the A ring. *Fusarium sporotrichioides* produces A-type trichothecenes, such as T-2 toxin or 4,15-diacetoxyscirpenol (4,15-DAS), that have either a C-8 hydroxyl or ester function or that lack a C-8 substituent, while *Fusarium graminearum* produces B-type trichothecenes, such as deoxynivalenol (DON), that have a carbonyl at C-8.

The biosynthesis of trichothecenes involves a complex pathway that begins with the sesquiterpene hydrocarbon trichodiene and consists of multiple oxygenation, cyclization, and esterification steps (7). A cluster of trichothecene biosynthesis genes has been identified in *F. sporotrichioides* (13). Within this cluster are two genes encoding P450 oxygenases, *Tri4* (12) and *Tri11* (1, 17); a sesquiterpene cyclase, *Tri5* (11); an acetyltransferase, *Tri3* (17); a pump, *Tri12* (2); and two regulatory genes, *Tri6* (22) and *Tri10* (25).

Two additional trichothecene biosynthetic genes, *Tri7* and *Tri8*, were recently described as part of a comparison of the *F. sporotrichioides* gene cluster and that of *F. graminearum* (6). *Tri7* controls C-4 acetylation in *F. sporotrichioides* (T. M. Hohn and S. P. McCormick, Abstr. 18th Fungal Genet. Conf., abstr. 16, 1995) but is a nonfunctional pseudogene in *F. graminearum* strains that produce DONs that lack C-4 hydroxyl or acetoxy groups (6, 15). In contrast, *Tri8* appears to be functional in both species (6, 15), but its role in trichothecene biosynthesis is much less clear. BLAST searches suggested that TRI8 was similar to lipases. A transformation-mediated gene disruption experiment of *Tri8* (6) produced a mutant strain, 8-5-6, that accumulated 4,15-DAS, which suggested that *Tri8* was involved in C-8 oxygenation. Feeding experiments with this DAS-accumulating mutant strain suggested that the gene might be involved in the addition of the isovalerate group to C-8. However, yeast transformed with *Tri8* did not metabolize neosolaniol, a likely substrate for C-8 esterification (6). Since both *F. graminearum* and *F. sporotrichioides* appear to have a functional *Tri8* but accumulate 15-acetyl-DON (15-ADON) and T-2 toxin, respectively, it seemed clear that *Tri8* was not solely involved with addition of the C-8 isovaleryl group. The similarity of *Tri8* in *F. graminearum* and *F. sporotrichioides* suggested that it encodes an enzyme related to one of the structural features that DON and T-2 toxin have in common. In order to determine its function, we disrupted *Tri8* in both *F. graminearum* Z3639 and *F. sporotrichioides* NRRL3299 and expressed *Tri8* in yeast.

MATERIALS AND METHODS

Strains and growth conditions. The *F. graminearum* wild-type strain, Z3639, was isolated from scabby wheat by R. Bowden (Kansas State University) (5) and was maintained on V-8 juice agar (24) slants. *F. graminearum* NA8b01, NA8b26, NA8b29, NA8b37, and NA8a02 contain disrupted sequences of *Tri8* as described below. The *F. sporotrichioides* wild-type strain was NRRL3299. *F. sporotrichioides* strains NA8-476, NA8-423, NA8-424, NA8-434, NA8-457, NA8-479, and NA8-480 contain a disrupted sequence for *Tri8* as described below. *F. sporotrichioides* 8-5-6 (6) and NA8-460 are hygromycin-resistant mutant strains that accumulate 4,15-DAS.

Medium and culture conditions. Mutant strains were maintained on slants of V-8 juice agar with hygromycin B (300 μg/ml). All fungal cultures were initially grown on V-8 juice agar plates under an alternating 12-h, 25°C light–12-h, 22°C dark cycle. Liquid cultures were grown on GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone; 20 ml in 50-ml Erlenmeyer flask). For toxin production, liquid cultures of *F. graminearum* were inoculated with a 20-mm mycelial plug cut from 1-week-old cultures grown on V-8 juice agar; liquid cultures of *F. sporotrichioides* were inoculated with spores (10^5/ml) washed from 1-week-old cultures grown on V-8 juice agar. For feeding experiments, liquid cultures of *F. graminearum* were inoculated with 10^5 conidia/ml harvested from mung bean cultures grown for 4 days at 28°C (4). All liquid cultures were grown at 28°C in the dark at 200 rpm. Rice cultures were prepared by inoculating rice in 2.8-liter Fernbach flasks with 2-day-old liquid cultures (11 ml/333 g of rice). The inoculated rice was incubated in the dark for 7 days at 28°C.

Gene disruption and transformations. To make the disruption vector for *F. graminearum Tri8* (GenBank accession no. AF359361), the gene was first amplified using *Pfu* polymerase (Stratagene, La Jolla, Calif.) with primers 1279 (5′-GTTCACTCACTCAGTATGGC-3′) and 1280 (5′-GAAATGGAAATTACCAGGC-3′) on a genomic template of Z3639. Amplification conditions were as recommended by the manufacturer (Stratagene) of the polymerase using a PTC-100 thermocycler (MJ Research, Watertown, Mass.). The resulting 1.3-kb fragment was band purified (UltraClean; MoBio, Solana Beach, Calif.) and cloned into the *Eco*RV site of pT7Blue-3 (Novagen, Madison, Wis.) using a blunt-end

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ligation (New England Biolabs, Beverly, Mass.) resulting in pFgTri8/pT7. For a selectable marker in fungal transformations, we used a chimeric hygromycin B phosphotransferase gene (*hygB*) containing promoter 1 from *Cochliobolus heterostrophus* (26). Using the restriction enzyme *Sac*I, followed by a fill-in reaction (Klenow fragment; New England Biolabs) (23), the P1-*hygB* region (approximately 2.5 kb) was cut out of pUCH4, a plasmid constructed in our laboratory. After UltraClean purification of the 2.5-kb fragment, a blunt-end ligation was performed with the prepared vector, pFgTri8/pT7, which had been cut with *Eco*47III, treated with calf intestinal alkaline phosphatase (New England Biolabs) to prevent self-ligation, and band purified. The *Eco*47III site is a unique site located approximately 500 bp downstream of the ATG start codon of *F. graminearum Tri8*. Thus, insertion of the P1-*hygB* gene into this site disrupts the coding sequence of *Tri8*. Since this latter step was a blunt-end ligation, the insert was capable of inserting in either direction and both orientations were found. Type *a* plasmids had the same direction of transcription as both the P1-*hygB* insert and *F. graminearum Tri8*, whereas type *b* plasmids had the insert in the reverse direction. The resulting plasmids, approximately 7.6 kb, were used to transform *F. graminearum* as previously described (21). For disruption in *F. sporotrichioides*, *F. sporotrichioides Tri8* (GenBank accession no. AF359360) was amplified, using the strategy outlined above and primers 1313 (5′-GCAAAGA GCCATTGATAGCTC-3′) and 1314 (5′-GACTACTTAAGGTGCAGAC-3′) on template FS3299. The 1.4-kb fragment was cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.). Again, the P1-*hygB* fragment was cloned into a unique *Stu*I site located approximately 560 bp downstream of the ATG start of *F. sporotrichioides Tri8*, yielding a disruptant plasmid of 7.8 kb in the *a* orientation. *F. sporotrichioides* NRRL3299 protoplasts were transformed with the disruption plasmid as described previously (21).

Disruption analysis. Fungal transformants were analyzed using PCR and Southern technique. In order to confirm *Tri8* disruption, primers outside the *Tri8* region were paired with primers located in the P1-*hygB* fragment. Since the *hyg* fragment could be in either the *a* or *b* orientation, transformants of *F. graminearum* with an *a* orientation were tested with 248 (5′-CTATGCCCTACAGCATC CAGG-3′) and 1283 (5′-CAGTTAATCCCTAGTCCATCGC-3′) and 247 (5′-G GTCAACATGATGTCAGG-3′) and 1285 (5′-CACCGTACGATCAGAGGC-3′). *F. graminearum* transformants using plasmids in the *b* orientation were tested with 247 and 1283 and 248 and 1285. All transformants were analyzed using 1279 and 1280. *F. sporotrichioides* transformants were screened using 1313 and 1314.

In the Southern analysis (23), genomic DNA of the *F. graminearum* and *F. sporotrichioides* wild-type and transformant strains was digested with *Bst*XI, blotted to a Nytran SuperCharge membrane (Schleicher & Schuell, Keene, N.H.), and hybridized to a ^{32}P-labeled probe (Prime-A-Gene; Promega, Madison, Wis.), consisting of 800 bp of coding region from *F. graminearum Tri8*. Primers 1312 (5′-GGACTCAATTCGCGCTGTTC-3′) and 1280 (5′-GAAATGGAAAT TACCAGGC-3′) on a template of cloned *Tri8* were used to make the probe.

Trichothecene toxin assays. Liquid cultures of *F. graminearum* and *F. sporotrichioides* were harvested after 7 days and extracted with ethyl acetate by vortexing in a conical tube, and the concentrated extract was analyzed by gas chromatography (GC) as described previously (16). Compounds were tentatively identified by GC/mass spectrometry (MS).

To isolate sufficient quantities of the compounds produced by the *F. graminearum* mutant strains for spectral analyses, we grew rice cultures (six cultures, 333 g each) of strain NA8b01 for 7 days. Cultures were extracted overnight with ethyl acetate. The concentrated extract was separated on a silica gel column eluted with 5% methanol–dichloromethane. The first four 100-ml fractions that contained 3,15-di-ADON and calonectrin were combined and further purified on a second column eluted with hexane-ethyl acetate (3:1); calonectrin (185 mg) was eluted in fractions 10 to 13; 3,15-di-ADON (290 mg) was eluted in fractions 15 to 18. The fifth fraction from the 5% column contained a mixture of 8-hydroxycalonectrin (20 mg) and 7,8-dihydroxycalonectrin (12 mg) and was further purified on a second column eluted with ether—the compounds coeluted after 250 ml in fractions 7 and 8. Compound identifications were confirmed by GC/MS and proton and carbon nuclear magnetic resonance (NMR).

In order to isolate sufficient amounts of the compounds produced by *F. sporotrichioides Tri8* mutants, GYEP cultures (six at 1,000 ml each) of NA8-476 were grown for 7 days and then extracted with ethyl acetate. The combined extracts were concentrated and then separated on a silica gel column eluted with 5% methanol in dichloromethane. Fractions (100 ml) were monitored by thin-layer chromatography and gas-liquid chromatography (GLC). Fractions 2 and 3 contained a mixture of 3-acetyl T-2 toxin (780 mg), 3,4,15-triacetoxyscirpenol (TAS) (570 mg), 3-acetyl buteryl neosolaniol (27 mg), and 3-acetyl propanyl-neosolaniol (30 mg). Fraction 5 contained 3-acetylneosolaniol (95 mg). Compound identifications were confirmed by GC/MS and proton and carbon NMR.

Chemical analyses. GLC measurements were made by flame ionization detection with a Hewlett-Packard 5890 Gas Chromatograph fitted with a 30-m fused silica capillary column (DB1; 0.25 μm; J&W Scientific Co., Palo Alto, Calif.). For routine screening of the trichothecene toxin phenotype, the column was held at 120°C at injection, was heated to 210°C at 15°C/min and held for 1 min, and was then heated to 260°C at 5°C/min and held for 8 min. Low-resolution mass spectra were obtained by GC/MS using the same temperature program with a Hewlett Packard 5891 mass-selective detector fitted with a DB-5-MS column (15-m by 0.25-mm film thickness). NMR spectra were determined in $CDCl_3$ on a Bruker WM-300 spectrometer with tetramethylsilane as an internal standard.

Expression of *F. sporotrichioides Tri8* and *Tri12* in yeast. The plasmid for *Tri8* expression in yeast was constructed by excising the *Tri8* coding region from pFgTri8/pT7 using the restriction enzyme *Eco*RI and by cloning the fragment into the *Eco*RI site of pYES2 (Invitrogen). This plasmid, pFgTri8/pYES2, was then transformed (8) into the yeast FsTri12p128/RW. The host yeast had *Tri12*, a gene encoding a trichothecene efflux pump, intercalated into the yeast genome (2); *Tri8* was added on an episome. We have previously used a similar construction for measuring episomal gene activity of other trichothecene biosynthetic genes (2).

The double transformant *F. sporotrichioides Tri8*/*F. sporotrichioides Tri12* was grown on supplemented glucose minimal media for 2 days at 28°C. Cultures were centrifuged, and the cells were resuspended in 1% yeast extract, 2% peptone, and 2% galactose to induce gene expression. Two hours after resuspension, seven C-3-acetylated trichothecenes were added in acetone solution: 3,4,15-TAS, calonectrin, 15-decalonectrin, 3,7,15-tri-ADON, 3,8-diacetylneosolaniol, 3-acetyl T-2, and isotrichodermin. As a control, the same seven trichothecenes were added to cultures of the wild-type yeast strain. A 2-ml aliquot was removed from each culture after 1, 2, 3, and 4 days and was extracted with 1-ml ethyl acetate with vortexing. Following centrifugation, the extract was analyzed for the substrates and their products by GLC and GC/MS as described above.

Whole-cell feeding. GYEP cultures of *F. graminearum* Z3639 and NA8b01 were initiated with 10^5 spores/ml produced on mung bean media (4). After 24 h, 3,4,15-TAS or calonectrin was added in an acetone solution. The final concentration of acetone in the cultures was less than 1%. Aliquots were removed and extracted with ethyl acetate at time points up to 48 h.

To determine if differences in pH accounted for the observed difference in the amount of hydrolysis between the wild-type and mutant strains, the pH values of 7-day-old GYEP cultures of Z3639 and NA8b01 were measured. In addition, C-3-acetylated compounds were incubated with culture filtrate from 2- or 3-day-old cultures to determine if extracellular enzymes were responsible for the hydrolysis of the C-3 acetyl group.

Cell-free system. Cell extracts of *F. sporotrichioides* NRRL3299 and NA8-476 and of *F. graminearum* Z3639 and NA8b01 were made from liquid GYEP medium cultures incubated for 42 h on a gyratory shaker (200 rpm) at 28°C in the dark. Cultures were vacuum filtered and washed with sterile water. Mycelia were collected on a filter, ground in a mortar with liquid nitrogen, and extracted with 3.5 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM 2-mercaptoethanol. The extract was centrifuged at 3,000 × *g* for 5 min. Assays were initiated by the addition of 100 μl of cell extract to a reaction mixture containing 250 μl of potassium phosphate buffer (pH 7.0), 100 μl of 20 mM $MgSO_4$, and 10 μl of substrate (1.5 mg/50 μl of acetone). Controls contained an additional 100 μl of potassium phosphate buffer in place of the cell extract. Reactions were run at room temperature. Immediately after addition of the cell extract and at 1, 2, and 4 h following addition, 100-μl aliquots of the reaction mixture were transferred to a glass vial containing 60 μl of ethanol. The samples were mixed on a vortex and then dried under a stream of nitrogen, redissolved in 150 μl of methanol, and analyzed by GLC. Seven C-3-acetylated substrates were tested: isotrichodermin, 15-decalonectrin, calonectrin, 3,7,15-tri-ADON, 3-acetyl T-2 toxin, 3,8-diacetylneosolaniol, and 3,4,15-TAS.

Trichothecenes. Isotrichodermin was isolated from cultures of *F. sporotrichioides* A11b (16) and 15-decalonectrin from *F. sporotrichioides* O2 (17). Calonectrin, 3,4,15-TAS, 3-acetyl T-2 toxin, 3,7,15-tri-ADON, and 3,8-diacetylneosolaniol were prepared by treating 15-decalonectrin, 4,15-DAS, T-2 toxin, 15-ADON, and T-2 tetraol, respectively, with pyridine and acetic anhydride.

RESULTS

Disruption of *Tri8* in *F. graminearum*. Disruption of *Tri8* was accomplished via transformation of wild-type *F. graminearum* Z3639 with plasmid pFgTri8a or pFgTri8b, which differed only in the orientation of the *hyg* gene (Fig. 1). Four of 41 hygro-

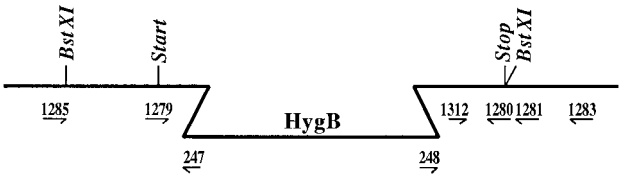

FIG. 1. Map of the *F. graminearum Tri8* region with the *hygB* insertion in the *a* orientation. Start and stop indicate the beginning and ending of the *F. graminearum Tri8* coding region. Primers and their orientation are listed. A probe used in Southern analyses was made by using primers 1312 and 1280 on genomic DNA.

mycin-resistant transformants with the *b* orientation and 1 of 11 hygromycin-resistant transformants with an *a* orientation accumulated three compounds not seen in cultures of the wild-type strain Z3639: 3,15-di-ADON, calonectrin, and 7,8-dihydroxycalonectrin (Fig. 2B). The remaining 47 hygromycin-resistant strains produced 15-ADON and a small amount of 3-decalonectrin and were indistinguishable from wild-type Z3639 (Fig. 2A).

The five transformants with the altered trichothecene phenotype were confirmed to be *Tri8* disruptants by PCR analysis with primers 1279 and 1280 (Fig. 3). The single fragment amplified from all five transformants was 2.5 kb larger than the

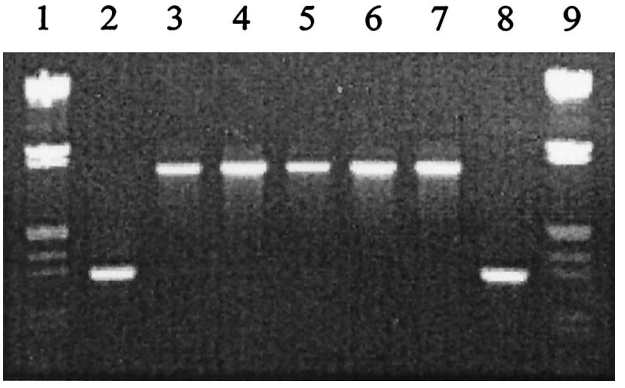

FIG. 3. PCR analysis using primers 1279/1280 on *F. graminearum* Z3639 and transformants. Lanes 1 and 9, lambda DNA cut with *Eco*RI-*Hin*dIII; lane 2, Z3639; lane 3, NA8a02; lane 4, NA8b01; lane 5, NA8b26; lane 6, NA8b29; lane 7, NA8b37; and lane 8, NA8b21.

single product (1.4 kb) amplified from the wild-type genomic DNA (Fig. 3). The increased size of the PCR products is consistent with disruption of *Tri8* via two homologous recombinational events between the plasmid and chromosomal *Tri8* sequences, since the *hyg* gene is 2.5 kb. If any of the transformants carried an ectopic integration of the transforming vec-

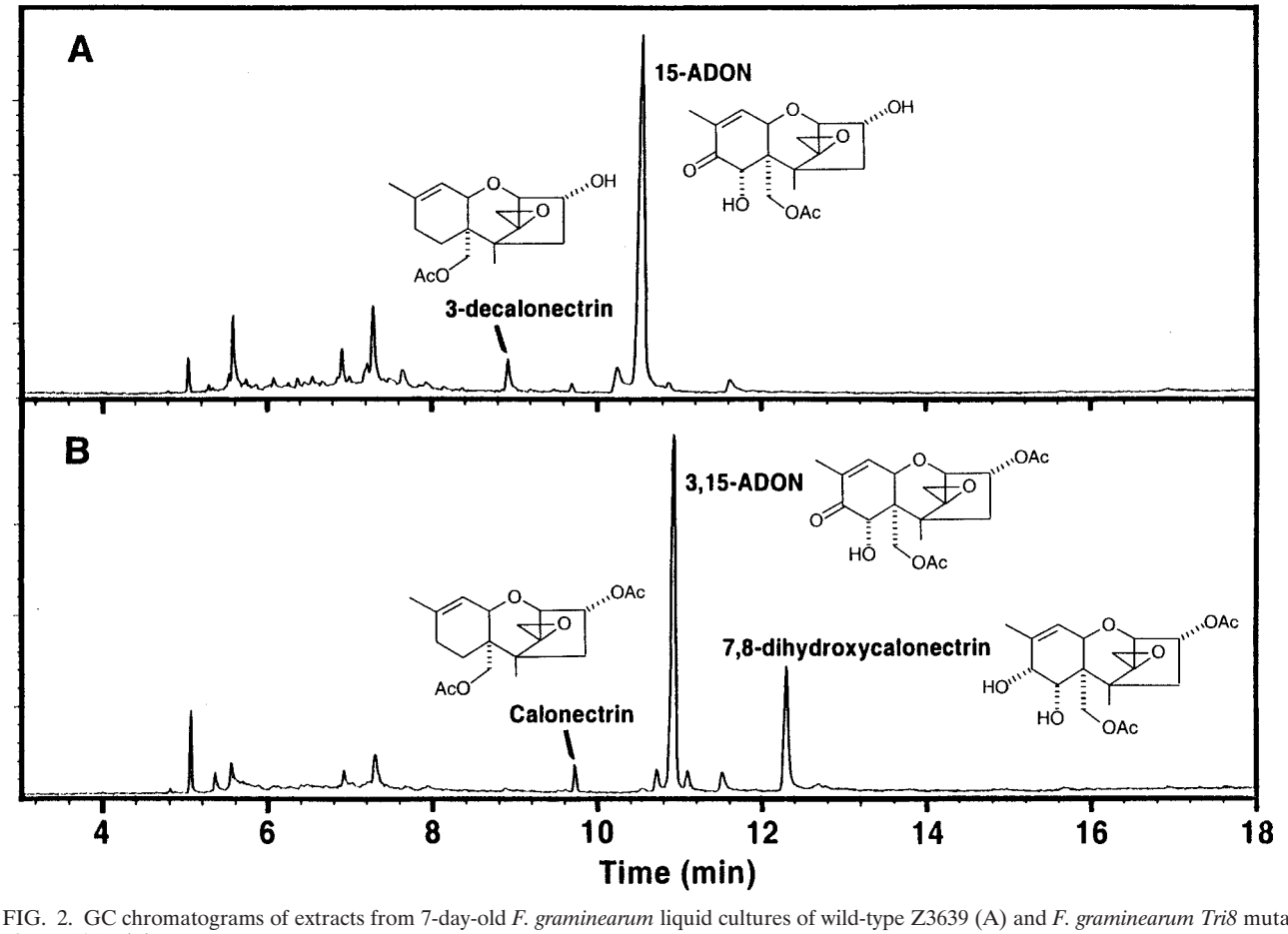

FIG. 2. GC chromatograms of extracts from 7-day-old *F. graminearum* liquid cultures of wild-type Z3639 (A) and *F. graminearum Tri8* mutant strain NA8b26 (B).

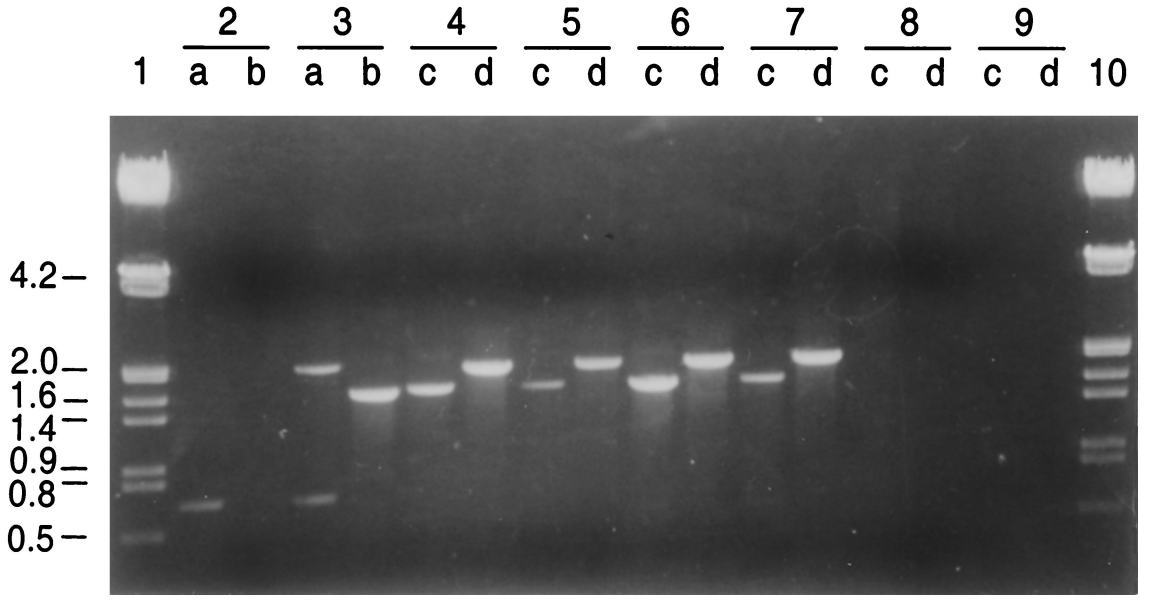

FIG. 4. PCR analysis on *F. graminearum* Z3639 and disruptants. Lanes 1 and 10, lambda DNA cut with *Eco*RI-*Hin*dIII; lanes 2 and 9, Z3639; lane 3, NA8a02; lane 4, NA8b01; lane 5, NA8b26; lane 6, NA8b29; lane 7, NA8b27; and lane 8, NA8b21. a, primers 248 and 1283; b, primers 247 and 1285; c, primers 248 and 1285; and d, primers 247 and 1283. Molecular size in kilobases.

tor, the PCR should amplify two bands, a 1.4-kb band corresponding to an intact *Tri8* gene and a 3.9-kb band corresponding to the disrupted gene. In the PCR of NA8b21 (Fig. 3, lane 8), which has a wild-type phenotype, the intact 1.4-kb fragment of *Tri8* is seen and no other band is apparent, suggesting ectopic integration of the hygromycin gene. To further prove that the *Tri8* gene was disrupted by a homologous recombinational event, PCR analysis using primers outside the *Tri8* region combined with P1-*hyg* primers (Fig. 1 for location of primers) showed that the five transformants produced the expected bands (Fig. 4). For example, transformant NA8a02 (Fig. 4, lane 3a), when tested with primers 248 and 1283, gave a dominant band of approximately 2 kb and a weak band of approximately 0.7 kb. The weak band was also seen in the wild-type Z3639 (Fig. 4, lane 2a), which suggests that this band is the result of mispriming. When the 5′ end of the gene was tested with primers 247 and 1285, a band of approximately 1.5 kb was seen in NA8a02 (Fig. 4, lane 3b), while no band was seen in the wild-type Z3639 (Fig. 4, lane 2b). Since the other transformants tested for Fig. 4 were in the *b* orientation, the 5′ end was tested with primers 1285 and 248 and gave a band of approximately 1.5 kb (Fig. 4, lanes 4c to 7c). Lanes 8c and d show the results of the primer pairs (for the *b* orientation) on a transformant, NA8b21, which had a wild-type phenotype. Lanes 9c and d show the results for the *b* primer pairs on the wild-type Z3639. No bands are seen in the last four lanes, showing that these primers did not mispair on the wild type and that, without a true *Tri8* disruption, no bands are formed.

The PCR results were confirmed by Southern analysis (Fig. 5). The wild-type *F. graminearum* gave a band of about 2.4 kb (Fig. 5, lane 1). Transformant NA8a02 (Fig. 5, lane 2) gave a band of about 5 kb, which would be the expected size if the *hyg* gene (2.5 kb) were inserted into the *Tri8* gene. The other larger bands in lane 2 may be explained by either incomplete digestion of the genomic DNA or multiple insertions of the plasmid, either in the *Tri8* region or ectopically. It is apparent that the 2.4-kb band seen in the wild type is not present in the transformant.

Disruption of *F. sporotrichioides Tri8*. Disruption of *F. sporotrichioides Tri8* was accomplished via transformation of wild-type *F. sporotrichioides* NRRL3299 with plasmid pFsTri8a. Fifty-nine hygromycin-resistant transformants were screened for production of trichothecenes. Fifty-one of the transformants had toxin profiles indistinguishable from that of the wild-type

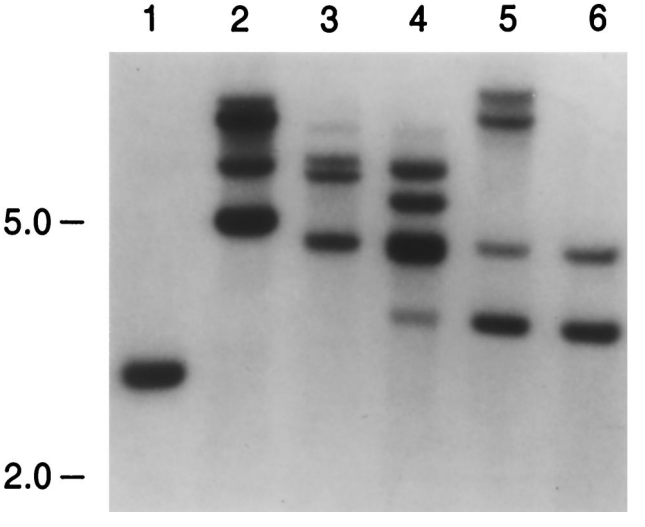

FIG. 5. Southern analysis of *Fusarium* wild type and transformants. Genomic DNA was cut with *Bst*XI and probed with a *Tri8* fragment. Lane 1, Z3639; lane 2, NA8a02; lane 3, NA8-423; lane 4, NA8-460; lane 5, 8-5-6; and lane 6, NRRL3299. Arrows indicate molecular size markers in kilobases.

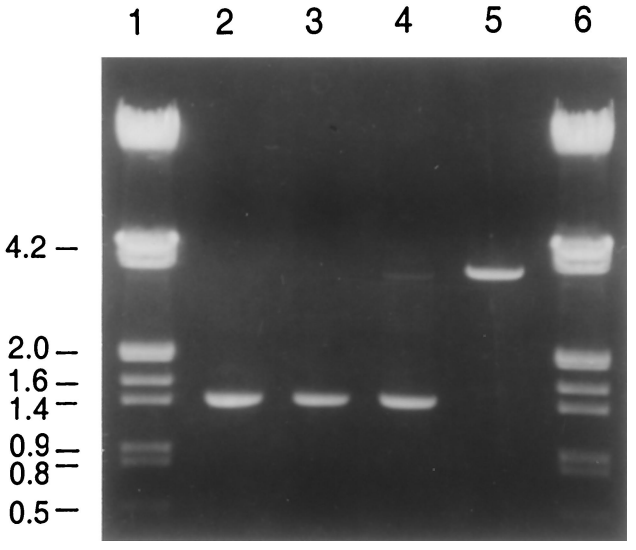

FIG. 6. PCR analysis of *F. sporotrichioides* wild type and transformants. Lanes 1 and 6, lambda DNA cut with *Eco*RI-*Hin*dIII; lane 2, NRRL3299; lane 3, 8-5-6; lane 4, NA8-460; and lane 5, NA8-423. Primers used: 1313 and 1314. Molecular size in kilobases.

NRRL3299, accumulating T-2 toxin, 4,15-DAS, and neosolaniol. Seven transformants produced trichothecenes not seen in cultures of the wild-type strain, including 3,4,15-TAS, 3-acetylneosolaniol, and 3-acetyl T-2 toxin. These seven strains were confirmed as *Tri8* disruptants by PCR (Fig. 6, lane 5). An additional hygromycin-resistant strain, NA8-460, produced 4,15-DAS in liquid culture, identical to the previously described strain 8-5-6 (6). PCR analysis of these two 4,15-DAS-accumulating strains indicated that *Tri8* was intact (Fig. 6, lanes 3 and 4). Southern analysis (Fig. 5) showed that, while the wild-type strain NRRL3299 (Fig. 5, lane 6) has two bands visible, the *Tri8* disruptant strain NA8-423 (Fig. 5, lane 3) lacks the lower band but has a band of increased intensity that is 2.5 kb larger. Both of the 4,15-DAS-accumulating strains, 8-5-6 (Fig. 5, lane 4) and NA8-460 (Fig. 5, lane 5), carry an intact *Tri8* band.

Whole-cell feeding. Whole-cell cultures of the wild-type strain *F. graminearum* Z3639 deacetylated both 3,4,15-TAS and calonectrin to 4,15-DAS and 3-deacetylcalonectrin, respectively. Whole-cell cultures of NA8b01 had no deacetylation of 3,4,15-TAS. Calonectrin added to cultures of the *F. graminearum Tri8* mutant was not deacetylated but rather was converted to 3,15-di-ADON.

Culture filtrates from 3-day-old cultures of Z3639 and NA8b01 that were incubated with 3,4,15-TAS had no measurable metabolism of the trichothecene. The pH of both the wild-type strain Z3639 and mutant strain NA8b01 became more acidic with the age of the culture. The pH of 7-day-old wild-type cultures was 3.6 and that of NA8b01 was 3.3.

***Tri8* expression in yeast.** *Tri8* was coexpressed with *Tri12*, which encodes a pump, because earlier experiments had shown improved conversion rates for two acetyltransferases, TRI101 and TRI3, when the acetyltransferase genes were coexpressed with *Tri12* (2, 18). *Tri8*/*Tri12* yeast was fed seven possible substrates with C-3 acetyl groups: 3,4,15-TAS, calonectrin, 15-decalonectrin, 3,7,15-tri-ADON, 3,8-diacetylneosolaniol, 3-acetyl T-2 toxin, and isotrichodermin. Five of the substrates, 3,8-diacetylneosolaniol, 3,4,15-TAS, calonectrin, 3-acetyl T-2 toxin, and tri-ADON, were completely converted to their C-3 hydroxyl analogs within 24 h. There was much slower deacetylation of 15-decalonectrin and isotrichodermin with only 5 and 15% conversion, respectively, after 3 days. None of the substrates was metabolized by the wild-type yeast strain.

Cell-free feeding. Cell-free deacetylation was tested with a number of substrates and cell extracts of *F. sporotrichioides* NRRL3299. Although there was rapid deacetylation at C-3 when 3,4,15-TAS, calonectrin, 3,7,15-tri-ADON, 3-acetyl T-2 toxin, or 3,8-diacetylneosolaniol was incubated with cell extracts, there was very little metabolism of isotrichodermin or 15-decalonectrin in 4 h. Cell-free preparations of *F. graminearum* Z3639 showed an identical pattern of substrate specificity but showed a relatively lower rate of C-3 deacetylation than did *F. sporotrichioides* extracts.

DISCUSSION

Both *F. sporotrichioides* and *F. graminearum* produce compounds with C-3 hydroxyl groups in liquid culture. *Tri101*, a gene lying outside the trichothecene gene cluster, encodes the trichothecene C-3 acetyltransferase (14). *Tri101* is both a biosynthetic gene (18) and a means of resistance or self-protection (14). Since TRI101 acts to acetylate the free C-3 hydroxyl group in *Fusarium* trichothecene biosynthesis (18), the ultimate production of toxins with C-3 hydroxyl groups requires an esterase to remove the protecting group. C-3 hydroxyl trichothecenes are significantly more phytotoxic than their C-3 acetyl analogs in a model *Chlamydomonas* system (3).

Gene disruption of *Tri8* in both *F. graminearum* and *F. sporotrichioides* resulted in strains that accumulate C-3 acetyl trichothecenes. These results strongly suggested that deacetylation at C-3 was blocked and that *Tri8* encoded an esterase. Although the Southern analysis indicated that more than one copy may have inserted into the genome of each species, the fact that the same phenotype is seen in disruptants from both *F. sporotrichioides* and *F. graminearum* indicates that the disrupted *Tri8* gene is causing the loss of esterase activity.

F. sporotrichioides esterases were partially purified and characterized by Park and Chu (19, 20). The esterase isozyme III, which had primarily C-3 esterase activity when assayed by conversion of 3-acetyl T-2 toxin to T-2 toxin, is a possible product of *Tri8*. Although some C-4 esterase activity was reported for isozyme III and although deacetylation of trichothecenes at both C-3 and C-15 has been reported in whole cultures of *Fusarium* spp. (27), our feeding experiments with yeast expressing *Tri8* and with cell extracts showed that the *Tri8* esterase was specific for the C-3 position. For example, one of the substrates, 3,8-diacetylneosolaniol, has acetyl groups at C-3, C-4, C-8, and C-15, but only the C-3 acetyl group was removed. Since whole cultures and cell extracts but not culture filtrates were able to deacetylate 3,4,15-TAS, the *Tri8* esterase is not extracellular.

A previous *Tri8* gene disruption experiment with *F. sporotrichioides* produced a strain, 8-5-6 (6), that accumulated 4,15-DAS. For reasons that are not clear, gene disruption experiments of *F. sporotrichioides* with at least three different genes

have sporadically produced strains that accumulate 4,15-DAS (data not shown). Examination by PCR and Southern analysis of two of these strains from *Tri8* gene disruption experiments, 8-5-6 (6) and NA8-460 (Fig. 5 and 6), indicated that the transformants carry an intact *Tri8.* It is possible that integration of the *hyg* gene complex elsewhere in the genome may have disrupted another gene that encodes the C-8 oxygenase. The Southern analysis indicated that insertions have occurred in different areas of the genome of 8-5-6 and NA8-460, so there may be more than one gene involved in this process.

Studies with synthetic derivatives and some natural products have demonstrated that the epoxide and the C-9, C-10 double bond are required for trichothecene toxicity (10). We have recently shown that the C-3 hydroxyl group is also a key component of the phytotoxicity of *Fusarium* trichothecenes (3). *Tri101* encodes the trichothecene C-3 transacetylase that provides self-protection or resistance during biosynthesis (14, 18), converting isotrichodermol to isotrichodermin. Biosynthesis proceeds from isotrichodermin through a series of C-3 acetyl intermediates. Only two early biosynthetic intermediates, isotrichodermin and 15-decalonectrin, were poor substrates for TRI8. Although *F. sporotrichioides* and *F. graminearum* accumulate C-3 hydroxyl trichothecenes such as T-2 toxin and DON, respectively, the accumulation of 3-ADON, calonectrin, and 7,8-dihydroxycalonectrin in *F. culmorum* (9) suggests that *Tri8* may be a pseudogene or that its expression may be significantly different in that species.

In summary, we report that *Tri8* encodes an esterase that removes the C-3 acetyl group from *F. sporotrichioides* and *F. graminearum* trichothecenes based on several lines of evidence. Strains of *F. graminearum* and *F. sporotrichioides* with a disrupted *Tri8* accumulate C-3-acetylated trichothecenes. Heterologous coexpression of *Tri8* and *Tri12* in yeast resulted in a strain capable of removing the C-3 acetyl group from several trichothecenes. Cell extracts of wild-type *F. sporotrichioides* and *F. graminearum* deacetylated some but not all C-3 acetyl trichothecenes. Both yeast and cell-free feeding experiments indicated that isotrichodermin and 15-decalonectrin were poor TRI8 substrates. We conclude that *Tri8* encodes an esterase responsible for removal of the C-3 acetyl group from trichothecenes as a final step in biosynthesis. Since C-3 hydroxyl trichothecenes are more phytotoxic (3), *Tri8* may be considered a toxicity factor.

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Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the products, and the use of the name by U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

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